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BRCA1, named after breast cancer gene 1, is a human breast-ovarian cancer susceptibility gene whose mutation, deletion or under-expression has been implicated in most breast cancer. To better understand the function of the crucial BRCA1 protein and specifically, to explore the hypothesis that BRCA1 is a DNA-binding protein, we have begun an exciting line of research to isolate genomic targets for BRCA1 protein binding. If our hypothesis is correct, this research may help discover the identity of genes whose expression is regulated by BRCA1 and thereby provide important new insights into BRCA1-mediated tumor suppression. To accomplish these goals, we proposed to use an immunopurification strategy to capture BRCA1-DNA complexes and test isolated DNA fragments for their ability to bind BRCA1 in vitro. During the first year of funding we have laid the groundwork for successive years by raising specific antibodies to BRCA1 that can be used for immunopurifcation of BRCA1 targets. Our antibodies are an important resource for our DOD-sponsored research and also for other avenues of BRCA1 research in my laboratory. In summary, we have developed specific antibodies to the BRCA1 protein which will be used to isolate, by immunopurification, likely targets for BRCA1 binding.

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INTRODUCTION

BRCA1 and breast cancer.

The subject of this review is BRCA1, so named because it was the first breast cancer susceptibility gene identified. Genetic epidemiologic studies estimate that the cancer risk associated with germline mutations of BRCA1 is 84% for breast cancer and 45% for ovarian cancer, by age 70 years (1). Molecular analysis of the BRCA1 gene has shown that this risk is due to mutations or alterations in the nucleotide sequence of BRCA1 that affect either the expression, function or synthesis of the full-length BRCA1 protein (2). The same studies have revealed that up to 10% of young women diagnosed with breast cancer, unselected for family history of disease, carry germline mutations of BRCA1 (3, 4), suggesting that the cancer burden due to this gene is not restricted to rare multiple case breast cancer families as first thought. Moreover, that BRCA1 is involved in the development of most breast cancer, not just the familial cases, is highlighted by the reduced expression of BRCA1 in sporadic tumors (5). Clearly then, understanding the normal function of the BRCA1 protein is an important goal in breast cancer research.

Function of BRCA1.

Clues to the biological function of BRCA1 have come from a search of the predicted amino acid sequence for functional domains. This type of analysis has revealed that BRCA1 contains an N-terminal RING-finger motif, a nuclear localization signal, a ten amino acid granin consensus and two copies of a domain, dubbed BRCT for BRCA1 C-terminal domain, that is possibly involved in forming protein-protein interactions. The RING finger motif, named after the RING1 gene, contains seven cysteines and a single histidine residue that are believed to fold into two zinc fingers, suggesting that this domain might be important in DNA binding (6). Many other proteins, most of them transcription factors, contain similar or related domains, and disease-causing mutations in the RING finger motif that specifically substitute conserved cysteine residues, highlight the importance of this domain for BRCA1 function (2). Immunofluorescence using monoclonal BRCA1 antibodies (7) and epitope-tagging of ectopically expressed BRCA1 (8) suggest that BRCA1 protein is located in the nucleus, and a nuclear localization signal has been located between amino acids 501 and 507 (9). The presence within the BRCA1 protein of a near-perfect granin consensus, suggesting that BRCA1 is secreted, remains controversial (10-12). Reports of BRCA1 protein staining in mammary ducts, as if it were being secreted, still appear in the literature (13) but much of this apparent controversy is probably related to the use of antibodies that have low affinity for BRCA1 and/or cross-reactivity with unrelated proteins (14). The BRCT domain, which contains two copies of a tandemly repeated sequence has now been found in more than 40 proteins, many of them having roles in DNA repair (11, 15). The precise function of this domain is unknown but, at least among some DNA repair proteins, it might be involved in forming protein-protein interactions (15). The BRCT domain in BRCA1 has been shown to bind CtIP, a protein identified by its association with the CtBP transcriptional co-repressor (16). Importantly, disease-causing mutations in BRCA1, within the BRCT domain, abrogate BRCA1-CtIP binding (16), suggesting that tumor suppression by BRCA1 takes place, at least in part, by regulating gene transcription.

The search for a function for BRCA1 has recently turned towards DNA repair with several lines of evidence implicating BRCA1 in the repair of DNA damage. The first is that BRCA1 interacts with RAD51, a known DNA repair protein, and is co-localized with RAD51 in meiotic cells, on synaptonemal junctions (17). This fits well with what we know about RAD51, since RAD51 is believed

to be important in double-stranded DNA break repair (18) and synaptonemal junctions are the complexes that form during recombination. Second, the location and phosphorylation status of BRCA1 changes in response to ionizing radiation: BRCA1 "nuclear dots" disperse and BRCA1 becomes phosphorylated (19, 20). Third, Brca1 nullizygous mice are embryonic lethal, express high levels of p21, and can be partially rescued by p21 or p53 null mutations, implying that they are DNA repair defective (21-24). Fourth and finally, Brca1 -/- fibroblasts are defective in transcription-coupled repair (TCR) (25), a special form of base excision repair that targets the transcribed strand of transcriptionally active genes (26). Importantly, BRCA1 is associated with RNA polymerase II (27), an important component of TCR. Although the evidence for BRCA1 having a role in DNA repair is mounting, no specific function has yet been assigned to this protein: it could be a component of the enzymatic machinery needed to repair DNA, but equally possible it could be a regulator of DNA repair gene expression. If it were the latter, it would make sense of all that we currently know about BRCA1, from its putative role as a transcription factor to its seemingly ever more likely role as a DNA repair protein.

Purpose of the proposed research.

The purpose of the proposed research, based upon the hypothesis that BRCA1 is a DNA binder, was to identify likely genomic targets for BRCA1 protein binding. To do this, we planned to use a novel immunopurification strategy (28-30) through which BRCA1-DNA complexes could be specifically selected from solubilized chromatin fragments using an antibody that recognizes only BRCA1. Isolated DNA fragments would then be cloned, sequenced and tested for their ability to bind BRCA1 protein in vitro. DNA fragments capable of binding BRCA1 would then be introduced into a reporter system to determine whether they are able to confer BRCA1-dependent regulation. The long term aim of the project, assuming that sequence-specific DNA binding could be demonstrated, was to determine the identity of the putative genes regulated by BRCA1; since isolated fragments were expected to be derived from the promoter regions of genes, subsequent studies (outside of the scope of the proposed project) would be necessary to determine which of the fragments were associated with transcribed sequences and to determine the identity of the genes.

To accomplish these aims we decided upon a two-pronged research strategy: firstly, to develop our own specific antibodies to BRCA1 to immunoprecipitate BRCA1-DNA complexes; and secondly, in case specific antibodies could not be developed, express in breast cells an epitope-tagged BRCA1 protein. At the time of our original grant application, no highly specific antibodies to BRCA1 had been produced, despite numerous attempts, and therefore the development of antibodies ourselves was high risk, but had the potential for high payoff. In fact, the antibodies that we have raised have proved to be very specific (see below) and are an important resource for breast cancer research in our laboratory. The expression of an epitope-tagged BRCA1 protein in human cell lines has been much more difficult to obtain. Previous laboratories have found that ectopic expression of BRCA1 inhibits cell proliferation (31) and stably-expressing transfectants have been hard, if not impossible, to produce. To overcome this problem, we proposed to express epitope-tagged BRCA1 under the control of a tightly regulated promoter; expression would be switched off during the transfection, selection and propagation of stably transfected clones, and switched on prior to cell harvesting and immuno-purification. Still, despite working hard on this part of the project, regulated BRCA1 protein expression in human cell lines has been difficult to achieve (see below). However, since we have developed our own specific antibodies to BRCA1, we have been able to proceed with our project as planned, while exploring new ways to obtain

the aforementioned cell lines which will become more important as likely genomic targets for BRCA1 are isolated.

Scope of the first year's funding.

The research performed in the first year of funding was expected to lay the groundwork for successive years; specifically, the development of antibodies to BRCA1 and the generation of cell lines expressing a regulatable BRCA1 gene. In the following sections I have described in detail the methods we have used to perform these tasks and the results of our experiments to date.

BODY

1. Development of highly specific BRCA1 antibodies.

Bacterial expression of BRCA1-fusion proteins.

To raise antibodies to BRCA1, segments of BRCA1 cDNA corresponding to amino acids 1 to 340 and 760 to 1312 were cloned into a bacterial expression vector. The reason for choosing these portions of BRCA1 for antibody production was that a splice variant of BRCA1, lacking most of exon 11, is reportedly expressed (9) and we wanted to generate antibodies that would detect only the splice form (antisera raised to residues 760-1312) and total BRCA1 (antisera raised to residues 1-340). PCR-generated DNA fragments were cloned into the pQE vector series (Qiagen Inc., Chatsworth, CA) such that over-expression of cloned cDNA fragments would generate his-tagged fusion proteins that could be purified by affinity chromatography using nickel-containing supports. Upon induction of gene expression, both BRCA1 expression clones yielded large quantities of fusion protein that were purified to homogeneity for antibody production.

Immunization of rabbits and testing for BRCA1 antibodies.

Prior to commencing this work, approval was obtained from the Institutional Animal Care and Use Committee (IACUC) and all work performed in accordance with university guidelines. Briefly, animals were immunized by mixing equal volumes of purified fusion protein (at ~ 1.0 μg/μl) with Freund's adjuvant, and 0.1 ml aliquots injected subcutaneously in 2 locations. For the initial immunization, Freund's complete adjuvant was used and thereafter, incomplete adjuvant. Pre-immune serum was collected prior to the first immunization and immune sera after booster immunizations, which were given monthly. Antibodies to BRCA1, tested for by the appearance of a 220 kDa protein on straight western blots of MCF7 whole cell lysates, were detected after about 6 months. Antisera R5599 and R5600 were raised to the N-terminal BRCA1 fusion (residues 1-340) and R5869 to the exon 11-derived fusion (residues 760-1312).

Evidence that the observed 220 kDa protein detected was BRCA1 is supported by the following (see Figure 1): 1. Each of three different antisera detected the same 220 kDa protein on straight western blots while pre-immune serum did not. 2. Each antiserum, but not pre-immune serum, efficiently immunoprecipitated in vitro transcribed-translated BRCA1 protein. 3. Immunoprecipitation with one antiserum, followed by immunoblotting with a second antiserum always detected only a 220 kDa protein, confirming that each antiserum detected the same protein. Pre-incubation of antiserum with the immunizing fusion protein, prior to immunoprecipitation, blocked precipitation of the 220 kDa protein. 4. Finally, immunoprecipitation with MS110, a monoclonal BRCA1 antibody, followed by immunoblotting with any of our antisera, detected the same 220 kDa protein, consistent with the protein

detected being BRCA1. Combined, these experiments provide strong evidence that the polyclonal antisera we raised detect full-length BRCA1 and can be used for immunoprecipitation experiments.

Our BRCA1 antibodies are highly specific.

To demonstrate the specificity of our antibodies for BRCA1, we performed immunoblots using 12% polyacrylamide gels, thereby keeping almost all cellular proteins on the blots. The previous blots were made from 6% gels since a low percentage acrylamide was necessary to resolve the large BRCA1 protein. As shown in Figure 2, each antisera detected only a 220 kDa protein demonstrating that we have generated important reagents for our BRCA1 research. These antibodies may now be used to isolate BRCA1-DNA complexes from whole cell extracts.

2. Regulated expression of BRCA1 in MCF7 cells.

The Tet-Off system.

The Tet-Off system was originally developed by Gossen and Bujard (32), and is the most widely used system for the regulated expression of cloned genes. The system is based upon the tetracycline-resistance operon of the Tn10 transposon which is negatively regulated by binding of the Tet repressor (TetR) to Tet operator sequences (tetO) in the absence of tetracycline. In the Tet-Off system, the first 207 amino acids of the repressor protein, which contains the DNA-binding domain, is fused to the transactivator domain of the herpes simplex virus, creating a hybrid protein (tTA) which behaves as a transcriptional activator when it binds to operator sequences. In the absence of tetracycline, tTA is able to bind tetO, activating transcription, while in the presence of tetracycline, tTA is no longer able to bind tetO and transcription is switched off. In the system supplied by Clontech (Palo Alto, CA), tTA is encoded on a plasmid (called Tet-Off) together with a neomycin resistance gene for selection of stable clones constituitively expressing tTA protein. The gene of interest is cloned on a separate plasmid (called pTRE), downstream of a tetracycline responsive element (TRE) and a minimal CMV promoter (P_{minCMV}). The TRE comprises seven copies of the 42 bp tetO sequence located upstream of P_{minCMV}. When tTA binds the TRE (in the absence of tetracycline), it activates transcription from the minimal CMV promoter driving high level expression of the cloned gene. In the presence of tetracycline, the transactivator is not able to bind the TRE and expression is switched off. For regulated expression in human cell lines, two consecutive stable transfections are therefore required: the first with the Tet-Off plasmid, and the second with the gene of interest cloned into pTRE

Generating MCF7 Tet-Off cell lines.

The MCF7 cell line was purchased from the American Type Culture Collection (ATCC) and a related cell line, MCF7/6 was obtained from Dr. Jim Freeman at the University of Kentucky. The MCF7/6 cell line is a derivative of MCF7, but the two have similar morphologies and grow at the same rate. The reason for using breast cell lines for these experiments is that biologically they are a relevant lineage and previous studies have demonstrated growth inhibition by BRCA1 in MCF7 cells (31). The Tet-Off plasmid, carrying the tTA transactivator, was transfected into MCF7 and MCF7/6 cell lines and stable clones selected in the presence of G418 (500 μg/ml). At least 30 independent clones were isolated for each cell line, cultured by passaging through dishes of increasing size and frozen in liquid nitrogen.

In order to test which of the cell lines were expressing tTA and might be suitable hosts for our experiments, we tested the ability of 10-12 clones for each cell line to regulate the expression of a

reporter gene, luciferase. Duplicate 60 mm dishes of cells were transfected with pTRE-Luc, one in the presence of tetracycline and one without tetracycline, and incubated for two days. The cells were washed once with PBS and 200 µl Reporter Lysis Buffer (Promega Corp., Madison, WI) was added to each dish. The cells were scraped from the dishes, transferred to 1.5 ml tubes, vortexed for 15 seconds and centrifuged (at full speed) for 1 minute. Cleared lysates were transferred to fresh tubes and luciferase assays performed by combining 5 µl of lysate with 50 µl luciferase substrate (Promega Corp., Madison, WI). Luciferase activity was measured immediately using a Turner Designs TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

The results of these assays are shown in Table 1. Approximately three-quarters of the cell lines tested had high levels of luciferase activity in the absence of tetracycline and low levels in the presence of tetracycline, with induction of luciferase expression ranging from 8 to over 400-fold. There was a great deal of variation in the actual luminometer measurements and presumably this occurred because some transfections had more cells than others, although I tried to plate out clones at the same cell density. For each cell line, I selected 4 or 5 clones that recorded the highest levels of luciferase induction and performed the transfections and luciferase assays once more to ensure that the cell lines were stably expressing the tTA protein and to determine the reproducibility of the induction. All of the cell lines showed high levels of luciferase expression, but one (MCF7/6 clone #8) was selected that had low basal expression and the highest level of luciferase activity upon induction of gene expression. We have used this clone during the past year for our experiments and have consistently seen 400 to 500-fold induction of reporter gene expression.

Construction of pTRE-BRCA1.

To construct the pTRE expression vector carrying epitope-tagged BRCA1 it was necessary to retrofit a BRCA1 cDNA clone that we obtained from Dr. Wen-Hwa Lee. We needed to add Sac II restriction sites to both ends of the cDNA for cloning into pTRE, and modify the 5' end of the coding sequence by adding a Kozak start site and an epitope tag. These modifications were made in two steps which were both performed by PCR-cloning: the first step being the 5' modifications (Sac II site, Kozak consensus and epitope tag) and the second being the 3' Sac II site.

For the first step, a PCR primer was designed that carried a SacII site, Kozak consensus, epitope tags for c-myc and 6xHis, and 21 base-pairs of BRCA1 coding sequence corresponding to amino acids 2-8 (Figure 2a). The purified primer was used in combination with a second primer, located downstream of a unique EcoRI site in the BRCA1 cDNA, to amplify a ~1 kb product using the full-length BRCA1 cDNA as the template. The PCR product was gel-purified, digested with SacII and EcoRI , and subcloned into equivalent sites present in the Wen-Hwa Lee cDNA. Recombinant clones were sequenced to ensure that no PCR-generated errors were introduced.

The second step was similar to the first; only this time we designed the 3' primer with a SalI site flanking the required SacII site (since the 5' end of the cDNA contains a SacII site) (Figure 2b) and utilized a unique Tth111I site located close to the end of the BRCA1 cDNA. A Tth111I-SalI fragment was cloned into the 5'-modified BRCA1 cDNA completing the retrofitting of the Wen-Hwa Lee clone. Again the 3' region containing the inserted fragment was sequenced to ensure that no PCR-generated errors were introduced into the cDNA.

Finally, a SacII fragment (Figure 2c), containing the entire BRCA1 cDNA, together with the aforementioned modifications, was inserted into pTRE, and recombinant clones checked for orientation by restriction mapping. Also, since the pTRE vector does not contain a selectable marker in human cells, we introduced a hygromycin-resistance cassette into a unique HindIII site located downstream of the inserted BRCA1 cDNA. The hygromycin cassette contains an enhancer-less promoter (so it should not interfere with BRCA1 expression) and was inserted in the same direction as BRCA1, eliminating the possibility that an antisense BRCA1 RNA could be expressed.

Isolation of MCF7 clones with regulated expression of BRCA1.

The MCF7/6-Tet-Off cell line (clone #8) that consistently gave high levels of reporter gene expression was transfected with pTRE-BRCA1. Clones were selected that could grow in the presence of G418 and hygromycin, and as before, propagated by serially passaging through cell culture dishes of increasing size. Importantly, during this process, tetracycline was present in the culture media at all times, to inhibit gene expression and prevent negative selection of BRCA1-expressing clones. For each clone, an aliquot of the cells was cryogenically frozen and the remainder plated into dishes for analysis of BRCA1 expression. Induction of BRCA1 protein was tested by removing tetracycline from the growth media (with careful washing of the cells with PBS) and incubation in tetracycline-free media for 48 hours, as we had done with the reporter construct. Cell lysates were prepared, assayed for protein concentration, and equal amounts of lysate loaded on polyacylamide gels. As a control, lysate prepared from the parental Tet-Off cell line was included on each gel and BRCA1 protein expression assayed by immunoblotting using our 5600 antiserum as the probe (examples are shown in Figure 4). To date, more than 50 independent clones have been analyzed in this way, but none have demonstrated over-expression of BRCA1. Some of the clones, such as 31, 39, 42 and 45 shown in Figure 4, appeared to have increased levels of BRCA1 but when the blot was repeated, using induced and non-induced lysate from the same clone, no over-expression of BRCA1 could be seen. We expected that about half of the clones would over-express BRCA1, some being negative owing to disruption of BRCA1 during integration, and that the level of induction would be in the range 2 to 10-fold. To exclude the possibility that induction of gene expression was occurring, but that BRCA1 protein level is tightly regulated, we analyzed some of the blots using antibodies to the c-myc and 6xhis epitope tags, but again did not see any difference between induced and non-induced lanes. We also performed northern blots, but could not see induction of BRCA1 mRNA expression in any of 10 clones tested. Our conclusion, based upon the evidence that we currently have available, is that there was selection against BRCA1-expressing clones, even though we were careful to include tetracycline in the growth media at all times.

The regulated expression of BRCA1 is important to achieve for a number of reasons. Firstly, if we can over-express 6xhis-tagged BRCA1 in human cells, it will enable us to purify full-length BRCA1 protein to perform gel shift assays. Immunopurification of BRCA1-DNA complexes is likely to identify numerous different DNA fragments and it will be important to screen these fragments, for example, by assaying their ability to bind BRCA1 protein in vitro, prior to further time-consuming testing. Secondly, we plan to test likely isolated DNA fragments for their ability to mediate BRCA1-dependent transcription by cloning them into a reporter system. The plasmid constructs will be transiently transfected into cell lines expressing different levels of BRCA1 protein. If we had a breast cell line in which we could modify BRCA1 expression, simply by altering the amount of tetracycline in the growth media, it would be an excellent host for these experiments. Lastly, if BRCA1 really is a transcription

factor, increased expression of this protein should alter expression of target genes in human cell lines. Thus, if we are able to develop cell lines in which we can regulate BRCA1 protein expression, they would enable us to test this hypothesis, once candidate target genes have been identified.

To pursue this aim further, we have begun experiments to express a different gene using the Tet-regulated system. Although this will not directly help our BRCA1 research, it will exclude the possibility that there was something wrong either with our Tet-Off cell lines or our pTRE vector. Again, we have repeatedly tested the MCF7/6-Tet-Off cell line that we have been using by induction of reporter gene expression; and restriction mapping of the pTRE vector and of our expression constructs, together with sequencing of the vector-insert boundaries in pTRE-BRCA1, have all revealed the expected results. If we obtain regulated expression of another gene, it will support our conclusion that there was negative selection against BRCA1-expressing clones. If this is the case we plan to do the following: 1. Select alternative Tet-Off cell lines for our experiments which are less "leaky." To date, we have used only one Tet-Off cell line which was chosen because it gave the greatest induction of reporter gene expression. Experiments in our laboratory and reports from other investigators at the University of Kentucky have found that the Tet-Off system is somewhat "leaky," meaning that a small amount of gene expression persists even in the presence of tetracycline. Possible options are to test alternative vectors, but before we do this, we plan on re-testing the Tet-Off cell lines that we have already developed, including some that have never been tested, to identify one which confers tighter regulation of reporter gene expression. A very reasonable compromise is a cell line which gives lower induction of gene expression, but tighter regulation in the presence of tetracycline. Such a cell line is expected to increase our chances of obtaining a stable clone with regulatable expression of epitope-tagged BRCA1. 2. If #1 is not successful, express BRCA1 by transient transfection, which should enable us to perform the reporter assays described above. For purification of recombinant protein to demonstrate protein-DNA interactions in vitro we may need to use bacterially-expressed protein since it is unlikely that we would be able to purify sufficient 6xhis-tagged BRCA1 from transient transfections of human cell lines.

CONCLUSIONS

We have generated during the past one year three different antibodies to the BRCA1 protein, two of them highly specific, detecting only full-length BRCA1 on immunoblots of whole cell extracts. In line with our Statement of Work, we have begun using these antibodies to isolate BRCA1-DNA complexes from extracts of human cell lines. Presently, we are optimizing conditions to increase the specificity of the selection procedure since other investigators who have used the same technique noted immunopurification of non-specific DNA fragments as a major obstacle. We are also testing formaldehyde cross-linking in our immunopurification procedure (34), since this technique stabilizes protein-DNA interactions by forming covalent linkages. As well, we plan to use each of our three different BRCA1 antibodies to make "libraries" of immunopurified DNA fragments: this should facilitate the identification of DNA fragments bound by BRCA1 in vivo, since true targets should be represented in each library. This work is currently ongoing in my laboratory.

A disappointment has been the lack of success in expressing epitope-tagged BRCA1. An original aim of our proposal was to use a regulated expression system to induce his-tagged BRCA1 expression, and purify BRCA1-DNA complexes using an antibody to the 6xHis epitope. The development of our own BRCA1 antibodies means that the cell lines are no longer needed for this purpose, but if we can

development them during the next year they will be useful in characterizing likely BRCA1 targets. For example, cell lines over-expressing 6xHis-BRCA1 may be used for purifying full-length BRCA1 protein for use in gel retardation assays; as hosts for reporter assays to determine if isolated DNA fragments may regulate gene expression; and in the future, to determine if up-regulation of BRCA1 affects expression of putative target genes in vivo. I have outlined, in the Body of this report, an experimental plan to develop these cell lines during the next one year of funding.

Finally, the development of antibodies to BRCA1 lay the groundwork for the isolation of BRCA1-DNA complexes, but also are an important resource for exploring other avenues of BRCA1 research. Specifically, we plan to use these antibodies in immunofluorescence to determine the pattern of BRCA1 expression in breast and ovarian tumors and in co-immunoprecipitation experiments to identify BRCA1-interacting proteins.

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Table 1. Luciferase assays in Tet-Off cell lines.

Clone #	Luciferase activity +tet	-tet	Fold induction
MCF			
4	13.7	3877	283
5	12.3	1496	122
8	2.8	992	354
9	65.1	31.8	0
14	6.2	3.4	0
15	22.2	30.2	1.4
16	2.7	4.8	1.8
17	1.6	172	108
19	6.2	1585	255
20	6.5	1702	262
21	11.0	1422	129
22	19.0	151.5	8.0
25	5.8	8.2	1.4
MCF7/6			
1	104	4208	40
8	17.0	7462	439
10	44.9	75.6	1.7
14	41.0	2673	65
15	46.4	7689	166
20	7.1	23.6	3.3
21	29.3	9289	317
22	70.0	2377	34
23	29.1	573	20
24	37.4	614	16
27	61.0	1452	24
28	11.4	516	45
30	82.1	11,663	142

Figure 1a. Immunoprecipitation of in vitro translated BRCA1

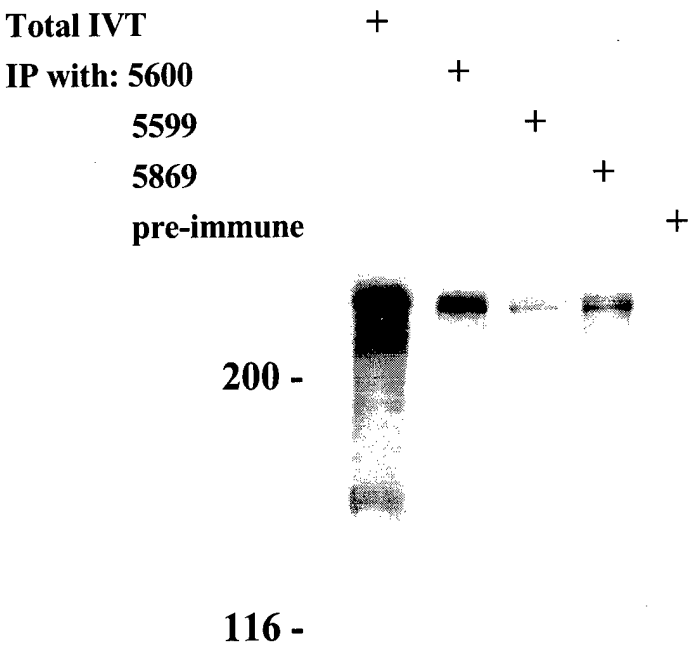


Figure 1b. Immunoblot probed with 5599

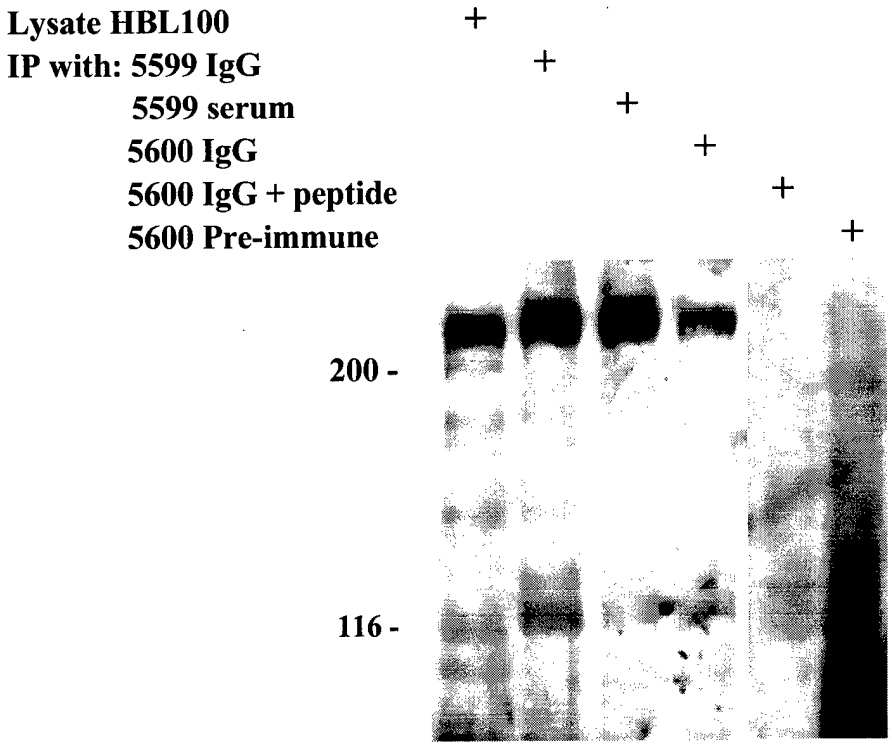


Figure 1c. Immunoblot probed with 5600

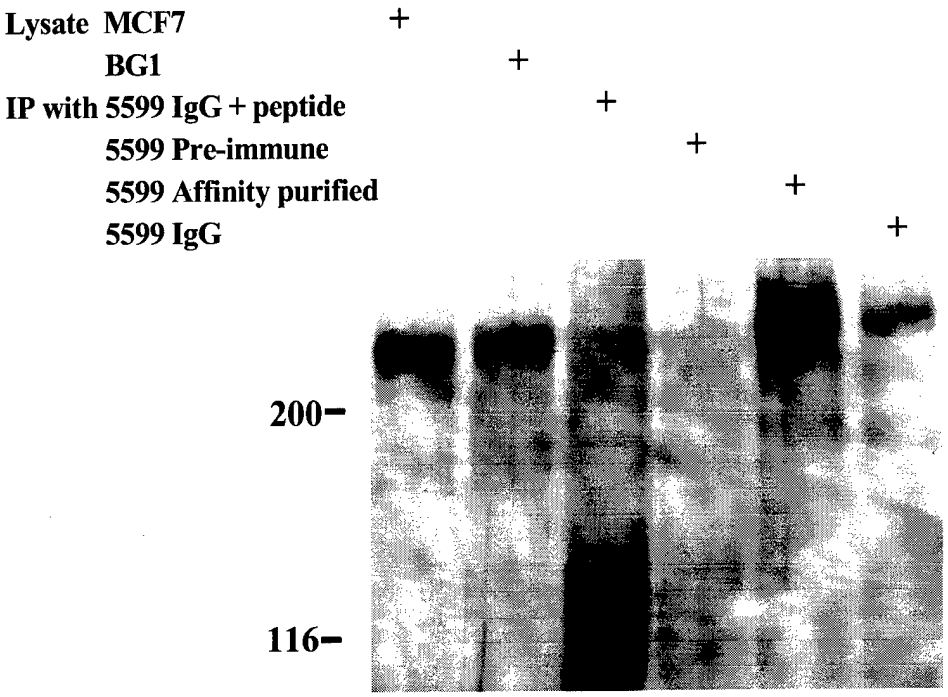


Figure 3d. Immunoblot probed with 5600

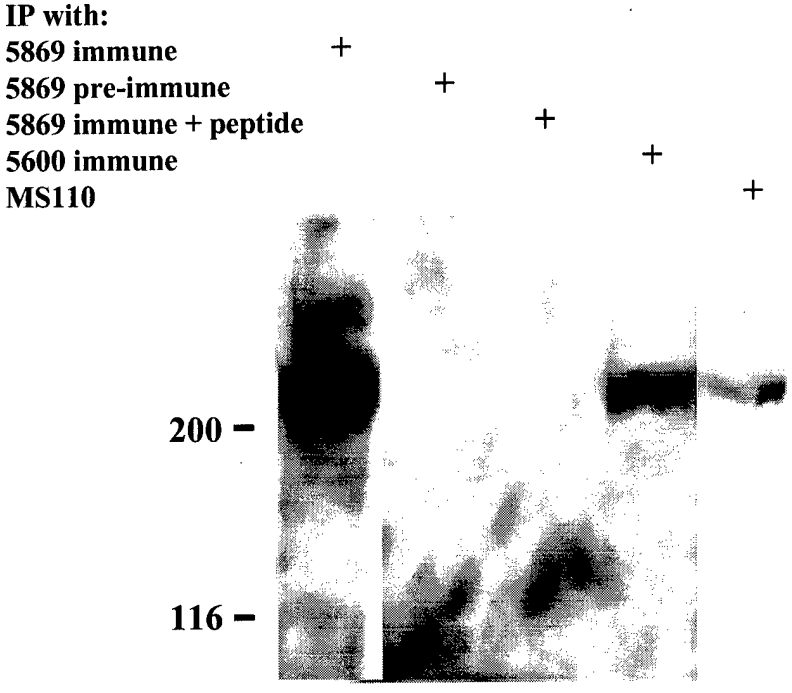


Figure 2. MCF7 lysates analyzed by immunobloting of 12% PAGE using our BRCA1 antibodies.

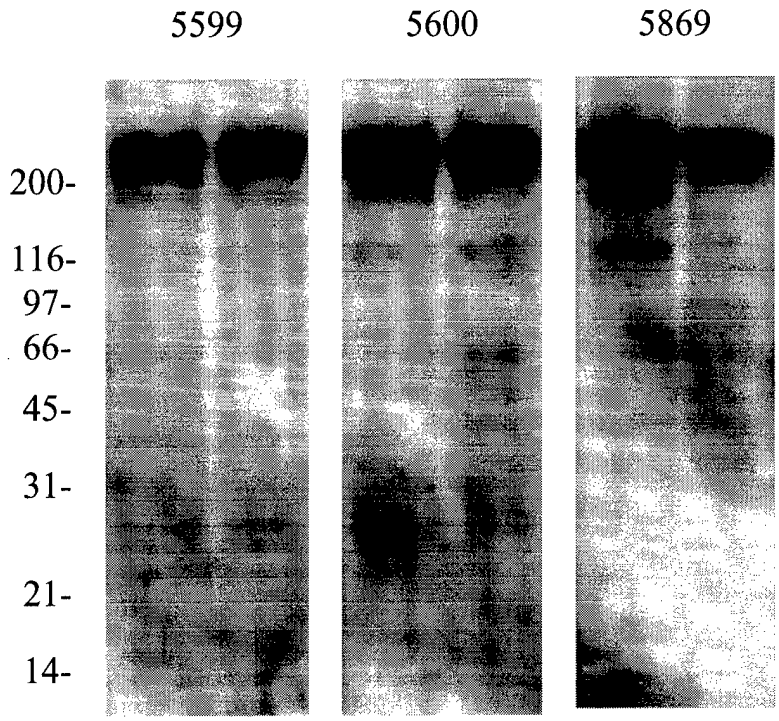
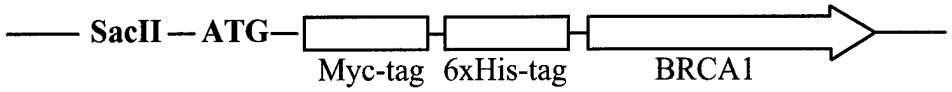


Figure 3. Retrofitting of the BRCA1 cDNA prior to cloning into pTRE.

a. Step 1: the 5' end.

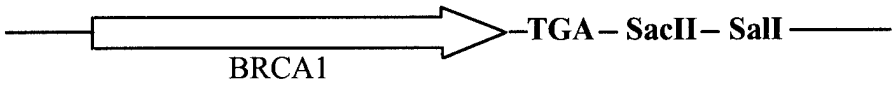


Sequence of the PCR primer

5'- GTCA - CCGCGG - ACCATG - GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG -
SacII Kozak Myc-tag

CAT CAT CAT CAT CAT CAT-GAT TTA TCT GCT CTT CGC GTT - 3'
6xHis-tag BRCA1

b. Step 2: the 3' end.



Sequence of the PCR primer

5'- TGAC - GTCGACCGCGG - TCA - GTA GTG GCT GTG GGG -3'
SalI SacII Stop BRCA1

Note: the sequence of the 3' primer is complementary to the BRCA1 sense strand.

c. Structure of the SacII fragment containing the retrofitted BRCA1 cDNA.

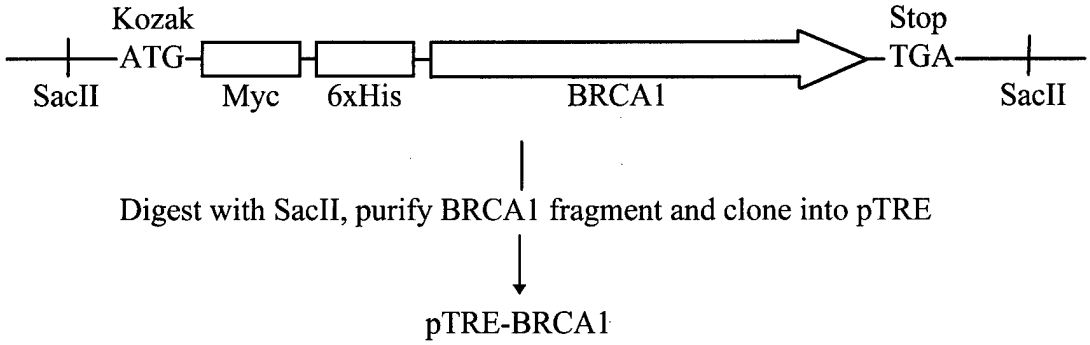


Figure 4. Testing of individual Tet-Off clones, stably transfected with pTRE-BRCA1, for up-regulation of BRCA1 protein expression.

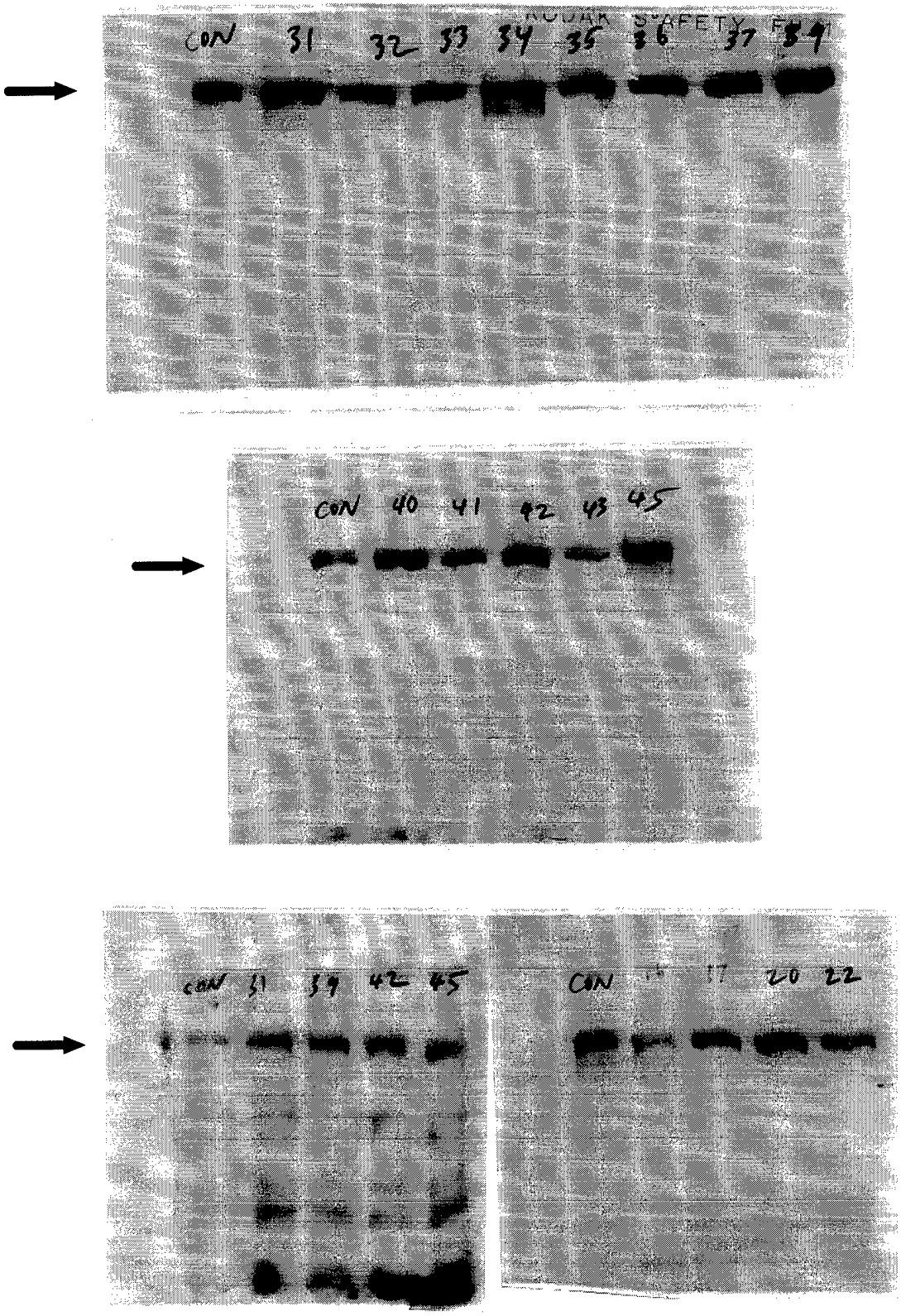


Figure legends.

Figure 1.
Figure 1a. Full-length BRCA1 cDNA was in vitro translated in the presence of ^{35}S-mthionine. An aliquot of the total reaction was analyzed in lane 1 (IVT) and radiolabeled BRCA1 protein immunoprecipitated using the indicated antisera.

Figure 1b. Lysates of the human breast cell line HL100 were analyzed either by straight western blotting (lane 1) or by IP-western (lanes 2-6) using the indicated antibodies. IgG was purified by affinity chromatography using Protein A-agarose beads and "peptide" indicates pre-incubation of IgG with immunizing BRCA1 fusion protein. The immunoblot was probed with R5599.

Figure 1c. As for Figure 1c, except that MCF7 and BG1 are breast and ovarian cancer cell lines, respectively. Immunoprecipitations were performed with MCF7 cell lysate and the immunoblot was probed with R5600.

Figure 1d. As for Figures 1b and 1c. Immunoprecipitations were performed using MCF7 cell lysates and MS110 is the BRCA1 monoclonal antibody raised by Scully et al. (7).

Figure 2. Lysates of MCF7 cells were analyzed by immunoblotting from 12% PAGE. A single blot containing many lanes of lysate was cut into strips and probed with each of our BRCA1 antibodies as indicated.

Figure 4. Analysis of BRCA1 protein expression in Tet-Off cell lines stably transfected with pTRE-BRCA1. The films represent immunoblots probed with BRCA1 antibody 5600. The arrows indicate the position of BRCA1; the lanes closest to the arrows on each blot contain lysate from the parental Tet-Off cell line; and numbers above each lane represent individual clones being tested for BRCA1 over-expression.